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Canada Centre for Mineral and Energy Technology | Centre canadien de la technologie des minéraux et de l'énergie

OBSERVATIONS ON THE MICROBIAL CELLULOSE-DEGRADATION PROCESS THAT DECREASES WATER ACIDITY

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November 1982

Project MRP-3.5.0.1.01
Control of Uranium Tailings Effluents

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44 pp.

MINERALS RESEARCH PROGRAM
MINERAL SCIENCES LABORATORIES
DIVISION REPORT MRP/MSL 84-32(TR)

MICROMEDIA

SEP 24 1984

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ABSTRACT

Pollution abatement of acid mine water might make use of a microbial cellulose-degradation process which promotes the growth of a heterotrophic microflora, including sulphate-reducing bacteria, by producing required organic nutrients and a pH-increase.

Several species of bacteria were isolated from wood decaying in an acidic mine-mill tailings area that neutralized the acidity of nutrient solutions having initial pH-values 3.5 to 5.0 and containing cellulose as sole source of carbon. Those bacteria were tentatively identified as members of the genera Aeromonas, Pseudomonas, Achromobacter, Flavobacter and Bacillus. Although they all grew in the presence of cellulose required for the decrease of water acidity, they were not cellulolytic. Cellulose breakdown was accomplished specifically by other distinct micro-organisms.

Chemical analyses of cellulose, after acidity-decrease, indicated that different processes took place in the pH-ranges 3.5 to 6 and above 6. In the low pH-range, chloride-uptake from the solutions by cellulose increased with the pH; cellulose permanganate-oxidation decreased and bisulphite-addition was negligible. In the pH-range above 6 only the bisulphite-addition was significant.

NOTES SUR LE PROCÉDÉ MICROBIEN DE DÉGRADATION DE LA CELLULOSE QUI DIMINUE L'ACIDITÉ DE L'EAU

par

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RÉSUMÉ

On pourrait diminuer la pollution des eaux acides d'exhaure en utilisant un processus microbien de dégradation de la cellulose qui favorise le développement d'une microflore hétérotrophe, notamment les bactéries sulfato-réductrices, en produisant des éléments nutritifs organiques et en élevant le pH.

Plusieurs espèces de bactéries ont été isolées à partir de bois en décomposition sur un site d'accumulation de résidus acides de broyage et d'extraction lesquelles ont neutralisé l'acidité de bouillons de culture dont le pH initial variait de 3,5 à 5 et qui renfermaient une seule source de carbone: la cellulose. Ces bactéries ont été provisoirement classifiées dans les genres Aeromonas, Pseudomonas, Achromobacter, Flavobacter et Bacillus. Bien que toutes se soient développées en présence de la cellulose requise pour la diminution de l'acidité de l'eau, elles n'étaient pas cellulolytiques. La cellulose s'est décomposée uniquement sous l'effet d'autres micro-organismes.

Les résultats des analyses chimiques de la cellulose effectuées après la diminution de l'acidité ont montré que différents phénomènes se sont produits lorsque les pH variaient de 3,5 à 6 et lorsqu'ils étaient supérieurs à 6. Dans le cas d'un pH faible, la cellulose captait de plus en plus de chlorure des bouillons à mesure qu'augmentait le pH; l'oxydation du permanganate de cellulose a diminué et la formation de bisulfite a été négligeable. Dans le cas d'un pH supérieur à 6, seule la formation de bisulfite était à signaler.

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INTRODUCTION

Acid water pollution from coal- and base metal sulphide-mining operations is due to sulphuric acid formed by the oxidation of pyrite or other sulphide minerals in the mine waste. Principles for successful abatement may be derived from natural processes counteracting the pollution not only in the waste but also in the water. Tuttle et al. (1,2) reported that the acidity and sulphate content of an acid mine water stream in Ohio was sufficiently decreased by seepage through a dam of decaying woodwaste to permit growth of sulphate-reducing organisms in the water downstream from the dam. These bacteria were able to use cellulose-degradation products as carbon source, and precipitated heavy metals as sulphides. Based on this observation Wakao et al. (3) studied the effects of various organic nutrients on sulphate reduction in acid mine water containing wood waste and concluded that sulphate reduction occurred only when the pH increased to more than 4. This coincides, generally with the limits of activity of sulphate reducers such as Desulfovibrio (4,5). The process of sulphate reduction was not separated from that of wood degradation in these experiments. Degradation products of either cellulose or of wood can be expected to provide organic nutrients for sulphate-reducing bacteria, but the mechanism increasing the pH to a value at which these organisms become active is obscure. High pH-values have been reported in connection with weathering of cotton fibres (6).

The effects of bacteria on cellulosic materials was studied at CANMET as part of a research project on the abatement of acid formation in uranium mine-wastes. This report contributes information about the pH-increasing process to aid in the development of possible practical pollution-abatement methods.

A number of bacterial species isolated from an acidic tailings area increased the pH in the presence of cellulose. These were not cellulolytic. The rate of pH-increase depended on the nature of anions in the water. However, the final pH-values in the presence of sulphate (the predominant anion in acid mine water) or chloride were not significantly different. The relationship between the analytically-sensitive chloride-uptake of cellulose and the pH was, therefore, determined. Chemical analyses of spent cellulose were done and the solutions of the cellulose suspensions were analyzed chromatographically to determine differences between pH-increasers and cellulose

hydrolyzers. Taxonomic properties of a few selected bacterial species were also determined to show the variety involved in the process.

Experiments on the process application are presently in progress.

MATERIALS AND METHODS

COLLECTION OF BACTERIAL SPECIES

A sample of decaying wood was collected from the acidic mine-mill tailings of Elliot Lake, Ontario. Two 10-g portions of the sample were each macerated in 500 mL saline (7) with a Waring blendor from which two-mL of each suspension were spread over agar and silica-gel plates (8,9) lacking cellulose. The plates were then covered with Whatman No. 1, 7.5 cm diam. filter paper discs and incubated for 30 days at 28°C.

Colonies that developed in the filter paper were subcultured by streaking needle samples on cellulose agar plates (8) containing the fungicides cycloheximide (4 mg/L), griseofulvin (1 mg/L) and pimafucin (1 mg/L) to prevent fungal growth from masking bacterial colonies. Cellulose for these media was prepared according to Hungate (10). When the number of species in a culture was reduced to three or four, according to different colony-morphology, needle samples were transferred to a medium(-I) containing 0.075% KH_2PO_4, 0.075% K_2HPO_4, 0.10% NH_4NO_3, 0.025% NaCl, 0.01% $MgSO_4 \cdot 7H_2O$, 0.001% $CaCl_2 \cdot 2H_2O$, 0.001% $FeSO_4 \cdot 7H_2O$, 0.0005% $MnSO_4 \cdot H_2O$, 0.001% yeast extract and 2% cellulose in distilled water. The pH was adjusted to 6.0 with 10% (v/v) H_2SO_4. One loopful of each suspension was streaked over medium-I agar plates for subculturing. The agar plates consisted of a layer of the medium containing 2% agar lacking cellulose, overlain with a thin layer of the complete medium containing 1.7% agar to facilitate observation of cellulose clearance. Subcultured agar-colonies or the solutions were used in the experiments. For the determination of changes in cellulose composition a starch medium (see Differentiation of Microbial Species "a") was used for the culture stock solutions. Due to significant differences in growth rate of species and their synergistic relationships, not all cultures used in the experiments were pure. They were selected according to different colony-morphology, ability to increase the pH of the medium or hydrolyze cellulose. Three fungal species obtained from the collection of the Eastern Forest Products Laboratory, Environment Canada were also

used in the experiments: A 419 Lenzites trabea, Pers. ex Fries; S 501 Polyporus versicolor, L. ex Fries; and D 47 Trichoderma viride. They had been received as slants, were subsequently grown in a 2% malt-extract solution and transferred to Erlenmeyer flasks containing the solution-I medium (pH 5.0) with either 2% cellulose or ball-milled wood.

DETERMINATION OF THE NUTRIENT pH-VARIATIONS PRODUCED BY BACTERIAL ACTION

a. A medium(-II) containing 0.03% KH_2PO_4, 0.10% K_2HPO_4, 0.10% NH_4NO_3, 0.10% NaCl, 0.02% $MgSO_4 \cdot 7H_2O$, 0.005% $CaCl_2 \cdot 2H_2O$, 0.002% $FeSO_4 \cdot 7H_2O$, 0.001% $MnSO_4 \cdot H_2O$, 0.15% Na_2SO_4, 0.05% yeast extract and 2% cellulose (10) in distilled water was used to determine the pH-increase produced by bacterial action. A loopful of each morphologically - different colony on agar medium-I was transferred to 250-mL Erlenmeyer flasks containing 100-mL solution-II to which the previously used fungicides had been added. The pH had been adjusted to 4.5 with 10% (v/v) H_2SO_4, and the flasks with the nutrient cellulose - suspensions had been autoclaved at 125°C for 20 minutes. The flasks were shaken in an "Environmental Incubator Shaker", Model G24, New Brunswick Scientific Co., at 200 rpm and 30°C. In a second experiment the pH-values were adjusted to 3.5 and the fungicides omitted. These media had also been sterilized at 125°C for 20 minutes before inoculation. During the experiments 1-mL samples of the cultures were aseptically removed from the flasks and transferred to 2-mL disposable sample-cups in which the pH was measured.

b. The method was repeated to determine the variations from pH 5.0 produced by the fungal species.

c. Medium-II without Na_2SO_4, adjusted to pH 3.5 with 10% (v/v) H_2SO_4 and containing either 0.6% cellulose or 1% glucose was used in the experiment establishing the use of cellulose, instead of glucose, for the decrease of water acidity.

d. A basal medium-III in the experiments about the effects of different anions contained (unless otherwise indicated) 0.02% $MgCl_2 \cdot 6H_2O$, 0.005% $CaCl_2 \cdot 2H_2O$, 0.002% $FeCl_2 \cdot 4H_2O$, 0.001% $MnCl_2 \cdot 4H_2O$, 0.05% yeast extract, 2% cellulose and 0.1% NH_4Cl in distilled water. Each of the test media was prepared by adding 0.13% of each of the following salts: $NaC_2H_3O_2 \cdot 3H_2O$, Na_2SO_4, KH_2PO_4, KNO_3 and NaCl to the basal medium. The pH-values were adjusted to 3.5 with the corresponding acids of the salts before autoclaving at 125°C

for 20 minutes. The cellulose was prepared by shredding Whatman No. 1 filter paper to less than 1 mm in a Wiley mill. The fungus Lenzites trabea and the bacterial culture No. 12-7-15 were grown with agitation at 30°C in duplicate Erlenmeyer flasks with 125 mL of each of these media, and the pH-values were monitored.

CELLULOSE-CHLORIDE RELATIONSHIPS

a. Culture No. 29-3-5 was grown in two 2-L Erlenmeyer flasks, each containing the basal medium-III plus 0.1% NaCl, 0.1% KCl, 0.5% (instead of 0.1%) NH_4Cl, and the cellulose replaced by either 0.2% or 0.4% Whatman No. 1 filter paper (-2 mm). The pH was adjusted to 3.5 with 10% (v/v) HCl; and the flasks were autoclaved at 125°C for 20 minutes. After inoculation the cultures were shaken with a Burrell "wrist-action" shaker at ambient temperatures for 14 d. The cellulose was then removed by filtration through an 11-cm diam. Whatman No. 50 filter paper disc, washed with a solution containing 1% Na_2CO_3 and 1% KNO_3 and dried at 70°C overnight. A modified ASTM method D-1161-60 (11,12) was used for the chloride determination. The results of the determinations were corrected for the weights and chloride contents of (fresh) cellulose and the washwater contained in the cellulose samples.

b. Six isolates were transferred from medium-I and each grown in 500-mL Erlenmeyer flasks containing 200 mL basal medium-III plus 0.325% NaCl, 0.325% KCl and 2% cellulose (Whatman No. 1, -2 mm), in an incubator shaker at 200 rpm and 30°C.

Eight other isolates were grown similarly but in 500 mL of the same medium in 1-L Erlenmeyer flasks at ambient temperatures using a Burrell "wrist-action" shaker. After 20 d, the pH and the cellulose-chloride concentration of each culture were determined as in the previous experiment.

PREPARATION OF SPENT-CELLULOSE

Isolates, selected for their fast pH-increase, were grown for 6 weeks in medium-II containing 4% cellulose (Whatman No. 1, -1 mm), lacking NaCl and having its pH adjusted to 3.8 or 4.5 with 10% (v/v) HCl. The pH of each suspension was measured and the cellulose was filtered off using a Whatman No. 52 filter paper disc. The filtrates were subsequently passed through a 0.2-µm

filter (Amicon Corp.), collected in sterile polypropylene bags, frozen and preserved for qualitative chromatographic analysis.

Chemically-oxidized cellulose samples were prepared by shaking 10 g cellulose (Whatman No. 1, -1 mm) in 300 mL 0.004 M-KIO_4 solution in a Burrell "wrist-action" shaker respectively for 4 h, 6 h, 24 h and 24 h plus subsequent heating to 85°C, and 24 h plus subsequent boiling for 1 h, before filtration.

All cellulose samples were washed with distilled water, air dried, stored in stoppered bottles at 8°C, and analyzed within 14 d. The sample weight used in the chemical determinations was converted to cellulose dried at 70°C overnight.

CHEMICAL ANALYSES OF SPENT-CELLULOSE

According to Hernadi (13) "for detecting changes (in degraded cellulose materials) the sensitivity of the (I.R.) spectroscopic determination is much weaker than that of applied chemical methods".

The following properties of each cellulose sample were, therefore, determined:

a. The addition of 0.005 N sodium bisulphite to the cellulose was determined by a method according to Feigl (14), quantitated for obtaining comparable results.
b. The quantity of a standard potassium-permanganate solution (0.005 N or 0.01 N) used for cellulose oxidation was determined by back-titration with a standard sodium-oxalate solution.
c. Carbonyl contents of the cellulose were determined using a modified hydroxylamine-hydrochloride method (15). The method had been standardized using vanillin ('Analar' B.D.H. Chemicals).
d. Carboxyl contents of the cellulose samples were determined using Yackel and Kenyon's calcium-acetate method (16). The end-point of the acid-base titration was indicated by a pH-meter. The method was verified using standard solutions of a few organic acids.

CHROMATOGRAPHY OF THE SOLUTIONS OF SPENT-CELLULOSE SUSPENSIONS

The filtrates obtained from the cellulose suspensions were concentrated ten times by evaporation. Ten-mL aliquots were further concentrated to 2-mL samples in centrifuge tubes using a Kontes tube-heater. Three fractions containing possible amino acids, organic acids and non-ionic substances, e.g., sugars, were obtained by passing the samples through Amberlite IR-120 and subsequently IRA-400 ion-exchange resin micro-columns (Glenco No. 3060 equipped with No. 3081-C). Each fraction was concentrated by evaporation to 1 mL of which 5 x 2 µl samples were applied to Whatman No. 1 chromatography-paper, and Whatman L5QD plates if "possibly positive" indications were obtained on paper.

For amino-acid chromatography a 1-butanol-pyridine-water, 1:1:1 solvent (17) was used in combination with a ninhydrin indicator (18). A 1-propanol-water-1-butanol-conc. NH_4OH-methylene chloride, 70:20:10:10:2 solvent (19) was used for thin layer chromatography with the same indicator.

For sugar paper-chromatography a 1-butanol-pyridine-water, 6:4:3 solvent (17) was used in combination with an amino-hippuric acid indicator (20), and for thin-layer chromatography a chloroform-methanol - 0.1 M lactic acid, 60:40:5 solvent (21) and an anisaldehyde indicator (22).

Two subfractions were obtained from the Amberlite IRA-400 column by elution with 2.5 and 6 N formic acid. These fractions were evaporated to dryness and redissolved in 1 mL water. For paper chromatography of organic acids a 1-butanol-pyridine-water, 6:4:3 plus 2% formic acid solvent (23) was used and Schweppe's reagent (24) or 0.03% morin (17) as indicators. For organic-acid thin-layer chromatography a 1-propanol-water-conc. NH_4OH-methylene chloride, 70:30:5:2 or an ethanol-methylene chloride-conc. NH_4OH-water, 70:40:20:2 solvent (25) was used with the same indicators. The chromatograms were compared with those of a blanc.

DIFFERENTIATION OF MICROBIAL SPECIES

The following methods were used to differentiate between species:

a. The purity of cultures growing in a medium containing 1% soluble starch, 0.15% trypticase (B.B.L.), 0.1% phytone peptone (B.B.L.), 0.05% yeast extract, 0.002% $FeSO_4.7H_2O$ and 0.001% $MnSO_4.H_2O$ in distilled water was checked by spreading different dilutions of each culture on nutrient

(Difco), trypticase-soy (B.B.L.) and starch (Difco) agar plates, and checking uniformity of cell morphology by microscope.

b. Needle samples of a colony of each starch-agar (Difco) plate were used for the Hugh and Leifson's test (26) on oxidative and fermentative breakdown of dextrose and for a motility test using Bacto's Motility Test Medium (27). A sample on the end of a 2-mm diameter sterile glass-rod was used each for Kovacs' oxidase test (28) and for a catalase test (29).

c. The remainder of the colony was transferred to 5 mL saline (7) in a 15 x 60-mm vial. A suspension was made using a "Vortex-Genie" rotating tube-shaker (Scientific Industries Inc.). This suspension was used for the determination of the cell morphology by microscope examination of methylene-blue stained slides under phase-contract and Nomarski differential-interference-contrast illumination, for observation of motility using the "hanging-drop" method and for Gram staining using Hucker's modification (30) and stain strips (Gugol Stain Co., Long Island City, N.Y.). Reported cell-sizes are the averages of 40 random measurements on photographs of the species grown on starch agar (Difco). The greatest part of the suspension was used for various tests contained in a diagnostic A.P.I. strip (31). The results of the tests were read after 48 and 72 h.

d. The carbohydrate tests in the A.P.I. strip were extended using stabs in tubes containing medium-II with 1% of the carbohydrate, 0.003% bromthymol blue and 1.7% agar, lacking cellulose and the pH adjusted to 6.7. The reactions were read after 48 and 72 h. Paper differentiation discs (32) could not be used because of the action of the species on cellulose. For the starch (amylose hydrolysis), acetate and malonate tests, and for the casein-hydrolysis respectively the same medium and sodium caseinate agar (BBL) were used. Growth on Difco's Pseudomonas Isolation agar is reported.

e. In addition to the sulphanilic acid, dimethyl-α-naphtylamine nitrate reduction test in the A.P.I. strip, Bacto nitrite test-strips involving the iodine-starch blue reaction (33) were used.

f. For flagella staining a suspension was made as under 'c' containing, however, a hundred times diluted saline and additionally 0.05% trypticase, 0.025% phytone peptone and 0.005% yeast extract. This was left overnight to obtain cells remaining suspended in the liquid medium. Five drops of a fixative consisting of (v/v 6:3:1) ethanol:chloroform:formaldehyde,

were added to the supernatant withdrawn from the suspension ten minutes before applying a loopful to a microscope slide. The slide had been cleaned by soaking in a "Liqui-Nox" soap solution and thorough rinsing in distilled water. The slide was dried using 'Kimwipes' Type 900L (Kimberley Clark of Canada, Ltd.) which produces glass surfaces on which a drop will run and spread. After air drying under the slight heat of a lamp, the mordant of Blenden and Goldberg (34) was used before silver-plating the flagella (35). On a second slide a BBL-modified flagella-stain of Leifson (36) was used. All staining solutions were filtered through a Whatman No. 42 filter paper directly before use.

g. A test for 2,3-butane-diol (37) was done because of the presence of Aeromonas and Bacillus species.

h. The use of poly-β-hydroxy butyric acid as sole source of carbon and energy was determined by observing the clearance of (0.007%) Nile Blue A (Harleco) halos around colonies on a 1.8% agar plate containing a modified mineral base of Stanier et al. (38) because of the presence of Pseudomonas species.

i. Reduction of 0.06% triphenyl-tetrazolium chloride (39) into formazan, in medium-II agar containing 2% cellulose (10) or 2% soluble starch was determined after 48 h.

j. Cellulose hydrolysis was indicated by the formation of clear halos around colonies on cellulose agar (medium-II lacking fungicides and cellulose covered with a thin layer of the complete medium) in 2 weeks and verified by the deterioration or breakage of a 5 x 120-mm Whatman No. 1 filter paper strip in 7 mL medium-II solution in a 16 x 125-mm culture tube in six weeks. The pH of both media had been adjusted to 6.5. Filterpaper disintegration in pH-3.5, 4.0, 4.5, 5.0, 5.5 and 6.0-environments by different cellulolytic species was also determined.

RESULTS

THE MICROBIAL ACTION ON THE NUTRIENT pH-VALUES

a. The results of monitoring the variations from the initial 3.5 and 4.5 nutrient pH-values produced by three cultures, selected for different pH-increase rates, are shown in Fig. 1. Approximately 30% of the twenty isolates did not increase the pH from 3.5. The variations from this value were more erratic than from the initial pH-4.5 value. They decreased often during the first ten to fourteen days before beginning the sustained increase. No pH-increase occurred in the absence of micro-organisms.

b. Figure 2 shows the pH-variations produced by the fungal species. The pH-decrease preceeding the final increase was not confined to initial pH-values below 4. The fungal species preferred wood over pure cellulose. However, cellulose as sole source of carbon was used in all other experiments to eliminate variables arising out of the complex composition of wood. The study focussed, therefore, also on the bacterial action.

c. The pH-variations of the solutions containing either cellulose or glucose as a carbon source, produced by two bacterial and fungal species, are shown in Fig. 3. Cellulose instead of glucose was used.

d. The average pH-values of the cultures containing different anions during microbial growth are shown in Fig. 4. Monovalent anions increase pH-values fastest. However, the differences between the final pH-values in the presence of chloride, nitrate or sulphate (the predominant anions in mine water) were not significant so that a chloride environment could be used in experiments determining the uptake of anions by cellulose. The determination of chloride in cellulose is analytically more sensitive than that of sulphate.

The action of bacteria responsible for pH-increase did not disintegrate filter paper. The action of cellulolytic species, however, did cause disintegration of filter paper which began at distinct pH-values for each isolate, as is shown in Table 1 (Differentiation method "j").

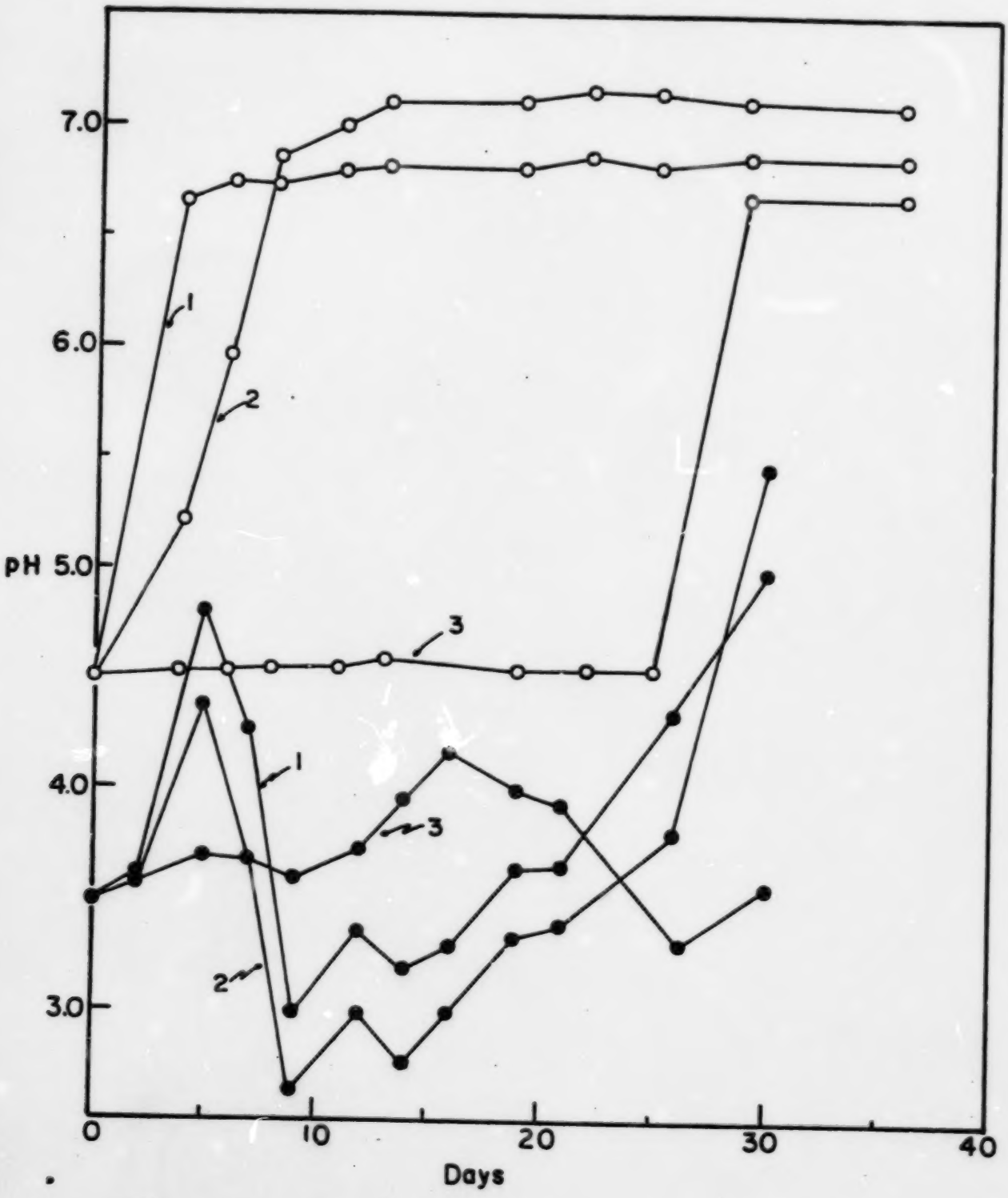

Fig. 1 - pH-variations in a nutrient containing cellulose as sole source of carbon, produced by the growth of three different bacterial cultures (1,2 and 3)

o - Initial pH of nutrient = 4.5
● - Initial pH of nutrient = 3.5

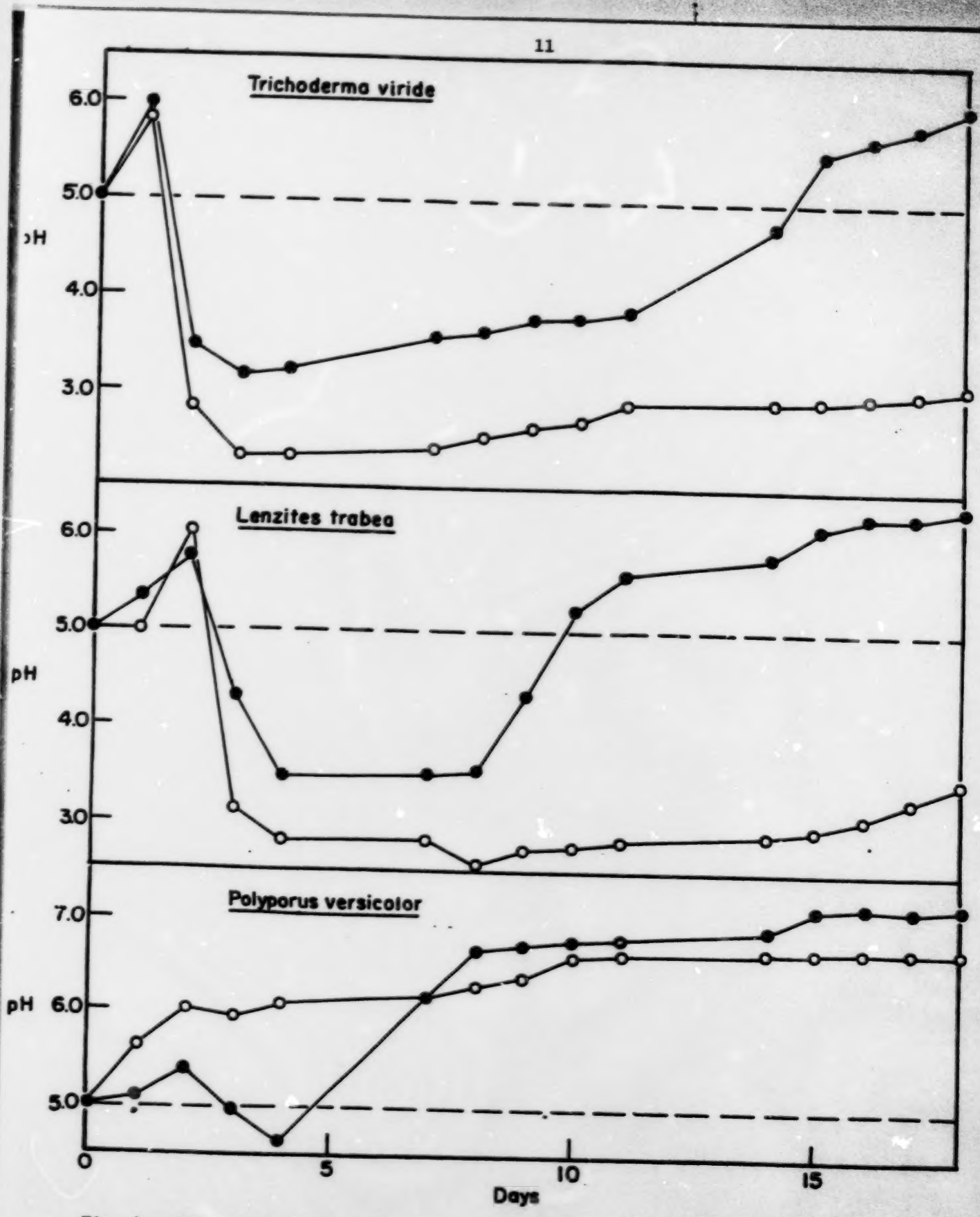

Fig. 2 - pH-variations in a nutrient containing either wood (•) or cellulose (o) as sole source of carbon, produced by the growth of selected fungal species

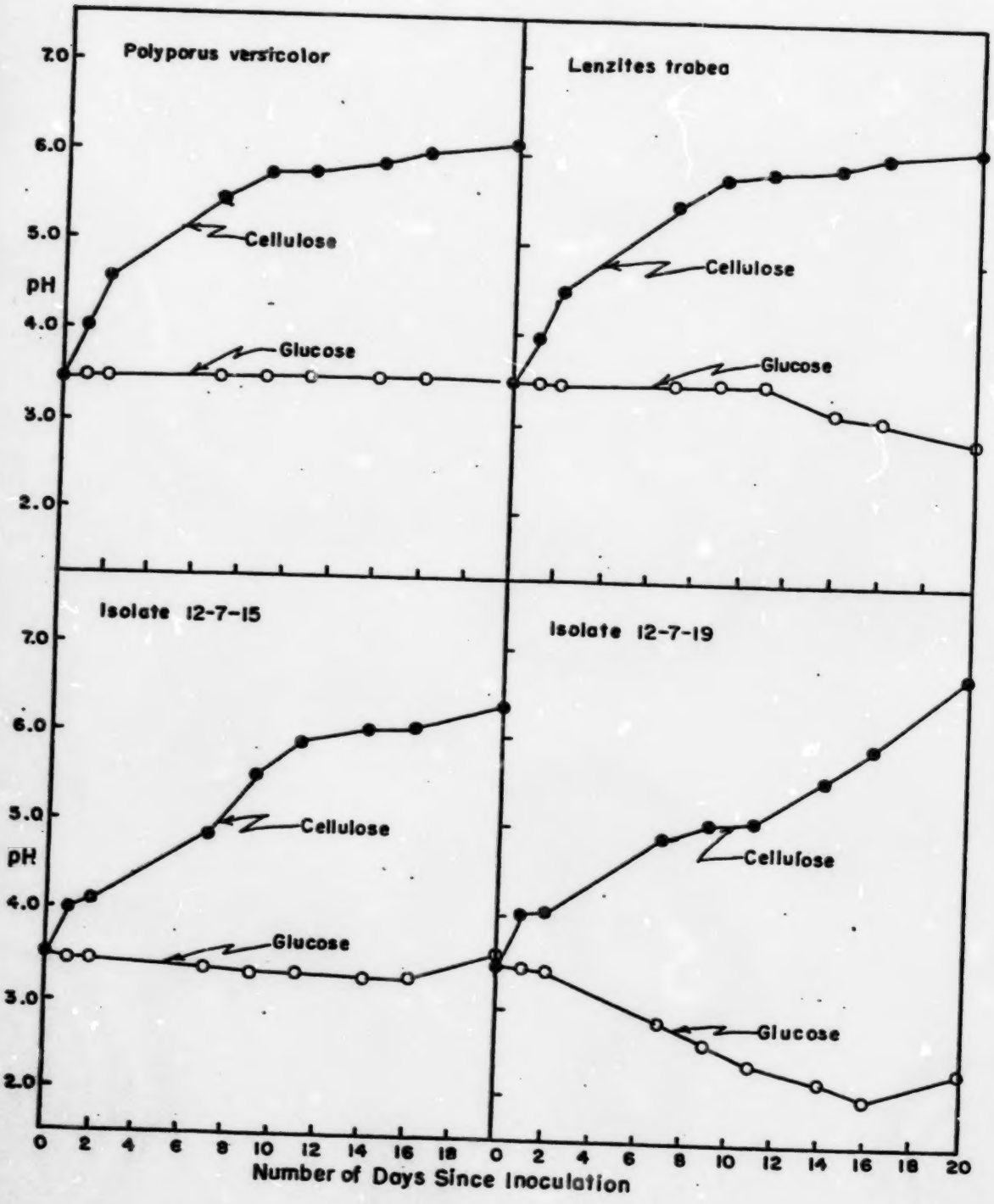

Fig. 3 - The effect of cellulose or glucose as sole source of carbon on the pH-variations produced by microbial growth

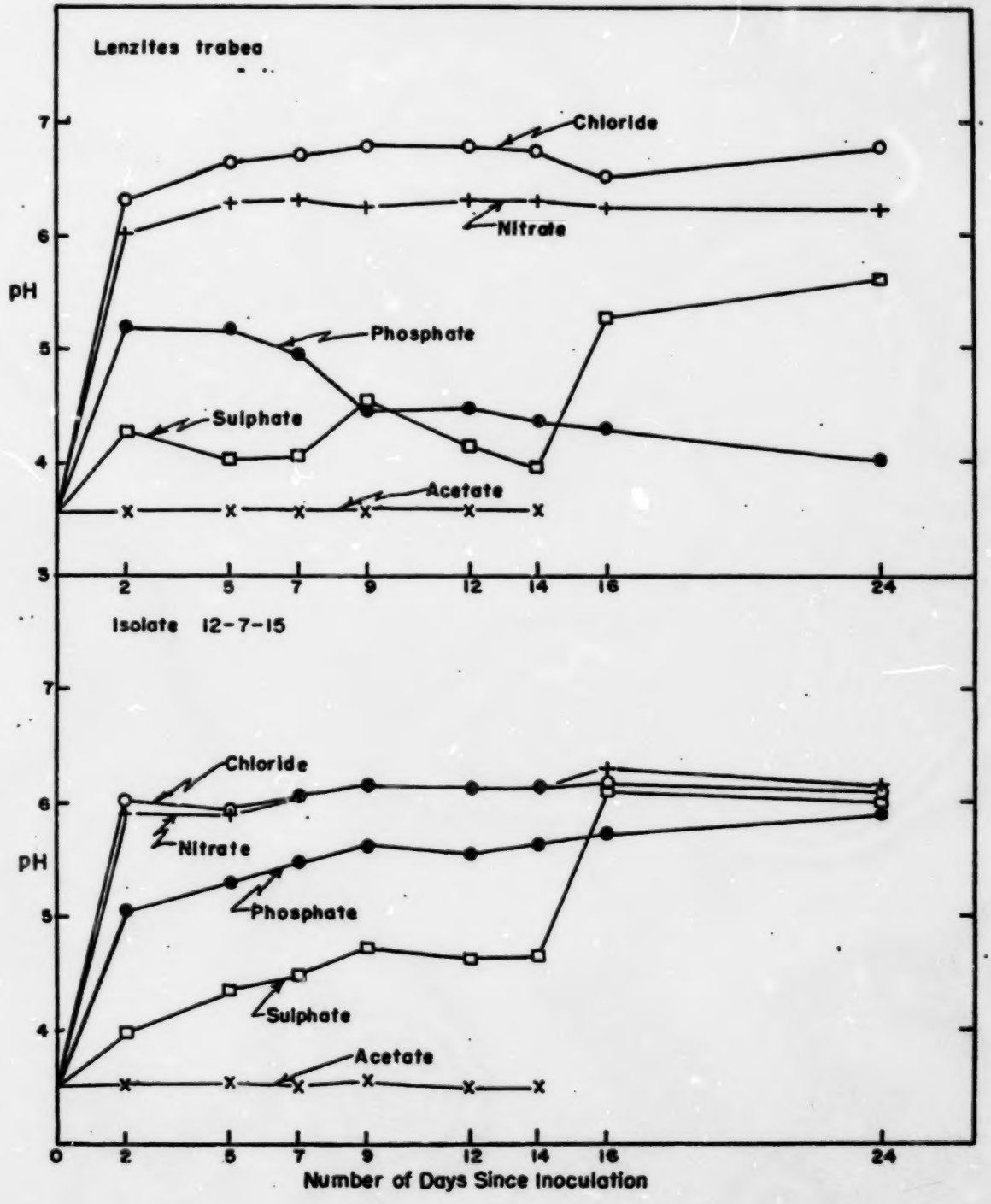

Fig. 4 - The effects of different anions on the pH-variations produced by two micro-organisms

Table 1 - Disintegration or breakage of filter paper by cellulolytic species after 6 weeks

Culture No.	pH 6.0	5.5	5.0	4.5	4.0	3.5
7-6(1)	+	+	-	-	-	-
7-6(3)	+	+	+	+	+	+
7-6(4)	+	+	+	-	-	-
7-6(5)	+	+	+	+	+	-
7-6(8)	+	+	-	-	-	-
7-6(9)	+	+	+	+	+	+
7-6(10)	+	+	+	+	-	-
7-6(15)	+	+	+	+	+	-
7-6(19)	+	+	-	-	-	-

+ Filterpaper strip broken or disintegrated

- Filterpaper strip not broken or disintegrated

CELLULOSE-CHLORIDE RELATIONSHIPS

a. Table 2 shows that the cellulose-chloride concentrations are inversely proportional to the cellulose concentrations indicating that the absolute amount of chloride removed from solution is independent of the cellulose concentration. In the presence of 0.2% cellulose 99.8 µg Cl/g cellulose was absorbed. This is approximately twice the concentration (46.5 µg Cl/g cellulose) absorbed in the presence of 0.4% cellulose.

The range of this relationship was not determined because of the following complexities indicated by the use of a number of different isolates.

b. The logarithmic relationships between the H^+-ion and the cellulose-chloride concentrations, shown in Fig. 5, consist of two ranges; pH 3.5 to, at least, 5.5 and higher pH-values up to approximately pH 8.5.

These results may depend quantitatively on the cellulose particle size, the oxygen-content of the water and the concentrations of the nutrient-chloride and cellulose. The relationship in the acid pH-range, however, does not seem to be affected by the action of different bacterial species.

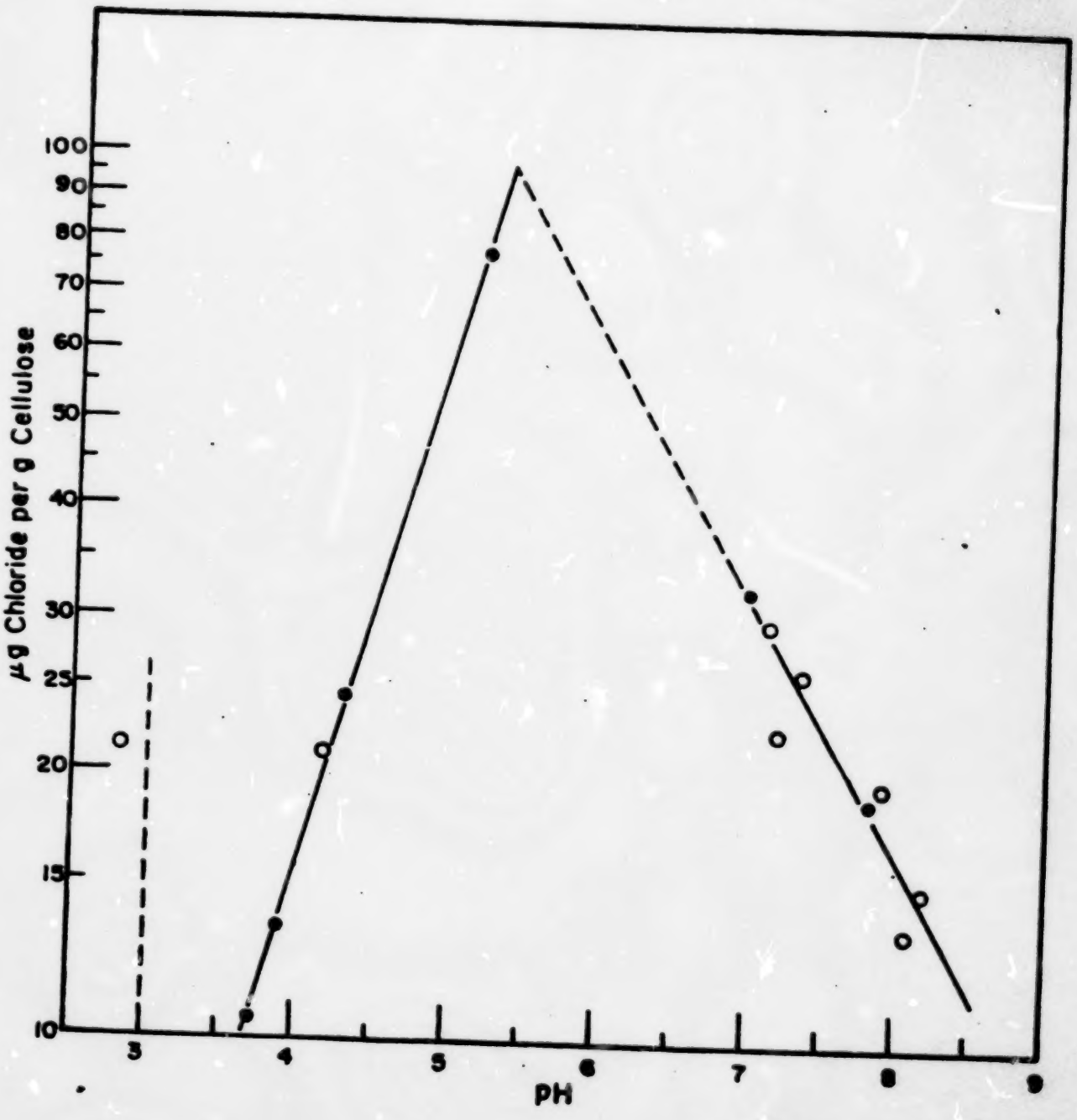

Fig. 5 - Relationships between the pH-values of the solutions and the cellulose-chloride contents

o - Cellulose-chloride contents of cultures grown in 200-mL solution
● - Cellulose-chloride contents of cultures grown in 500-mL solution

Table 2 - The effects of cellulose concentrations on the cellulose-chloride content

Culture	Cellulose wt %	pH	µg Cl per g cellulose
"29-3-5"	0.2	7.48	99.8
"29-3-5"	0.4	7.22	46.5

CHEMICAL ANALYSES OF SPENT-CELLULOSE

a. The results of the determinations of the bisulphite additions which is an indication of the concentration of aldehyde (C=O), or ketone (C=O) groups associated with the cellulose, are shown in Fig. 6. Those to cellulose used by pH-increasers above pH 5.9 were similar to the periodate-oxidized cellulose samples. All other values were negligible because they were similar to those of fresh cellulose or the cellulose control as indicated by the horizontal dashed line.

b. The permanganate oxidation of the cellulose samples indicating the degree of oxidation (or reduction) of the cellulose, is shown in Fig. 7. Oxidation occurred at pH-values lower than 5.9. The values obtained for cellulose hydrolyzers were significantly higher (indicating that the cellulose exposed to these bacteria were less oxidized, or more reduced) than those for the pH-increasers. Negligible values were lower than 10 mEq $KMnO_4$/100 g cellulose because of the value of fresh cellulose (indicated by the horizontal dashed line).

c. The results of the determinations of the carbonyl (all C=O) contents of the cellulose are shown in Fig. 8. As expected, cellulose exposed to bacteria exhibited similar or higher values than those of the bisulphite additions but did not show any pattern. Carbonyl values of periodate-oxidized cellulose increased according to the severity of the treatment.

d. The carboxyl (-C-OH) contents did not show any pattern either (Fig. 9). However, they were all lower than 1.5 mEq COOH/100 g cellulose and considered negligible except for those of the cellulose hydrolyzers.

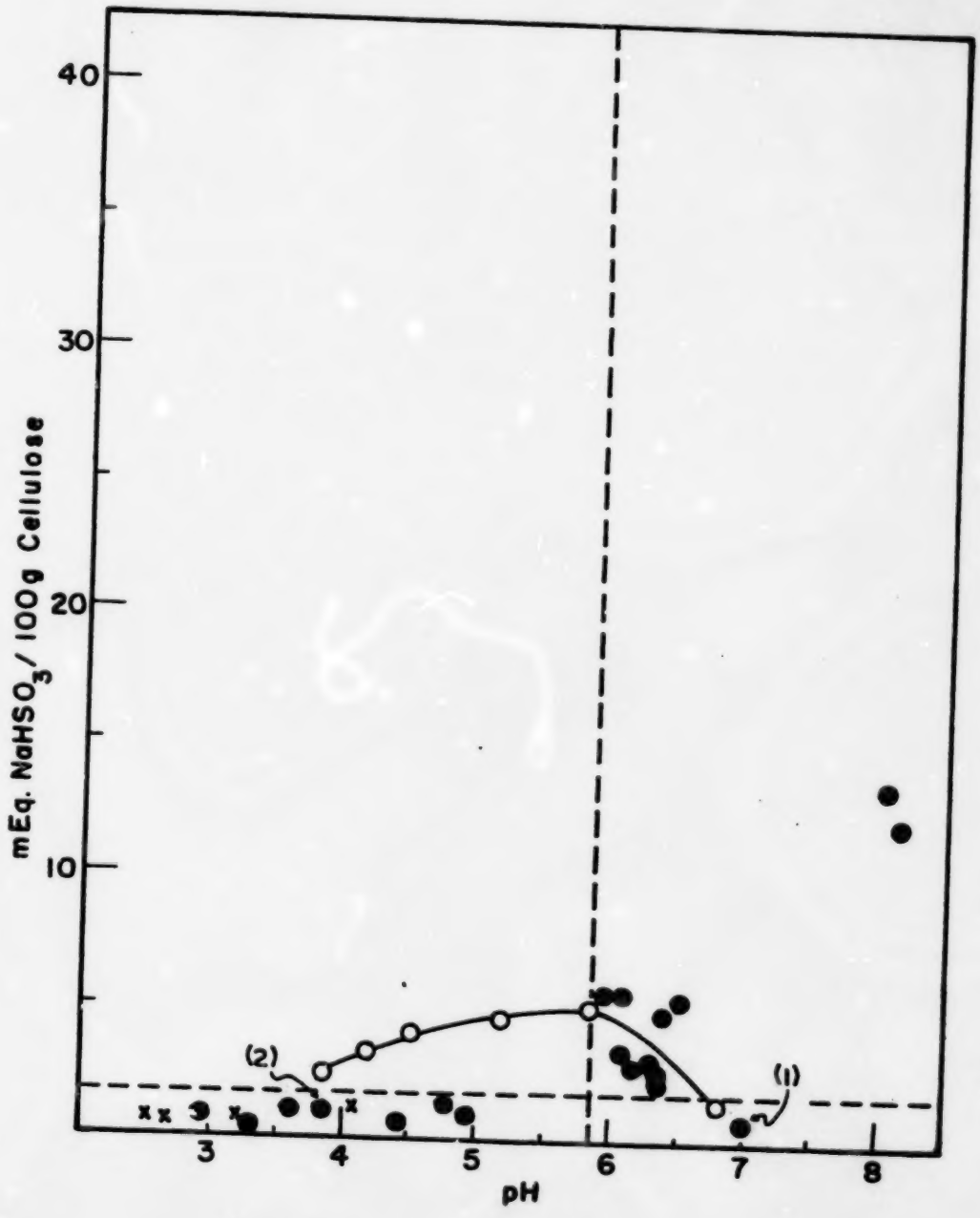

Fig. 6 - Bisulphite-addition to cellulose

- ● - pH-Increasers
- x - Cellulose hydrolyzers
- o - Periodate oxidation

(1) - Fresh cellulose
(2) - Cellulose control

Dashed line at pH 5.85 separates significant bisulphite-addition values from the control range indicated by horizontal dashed line.

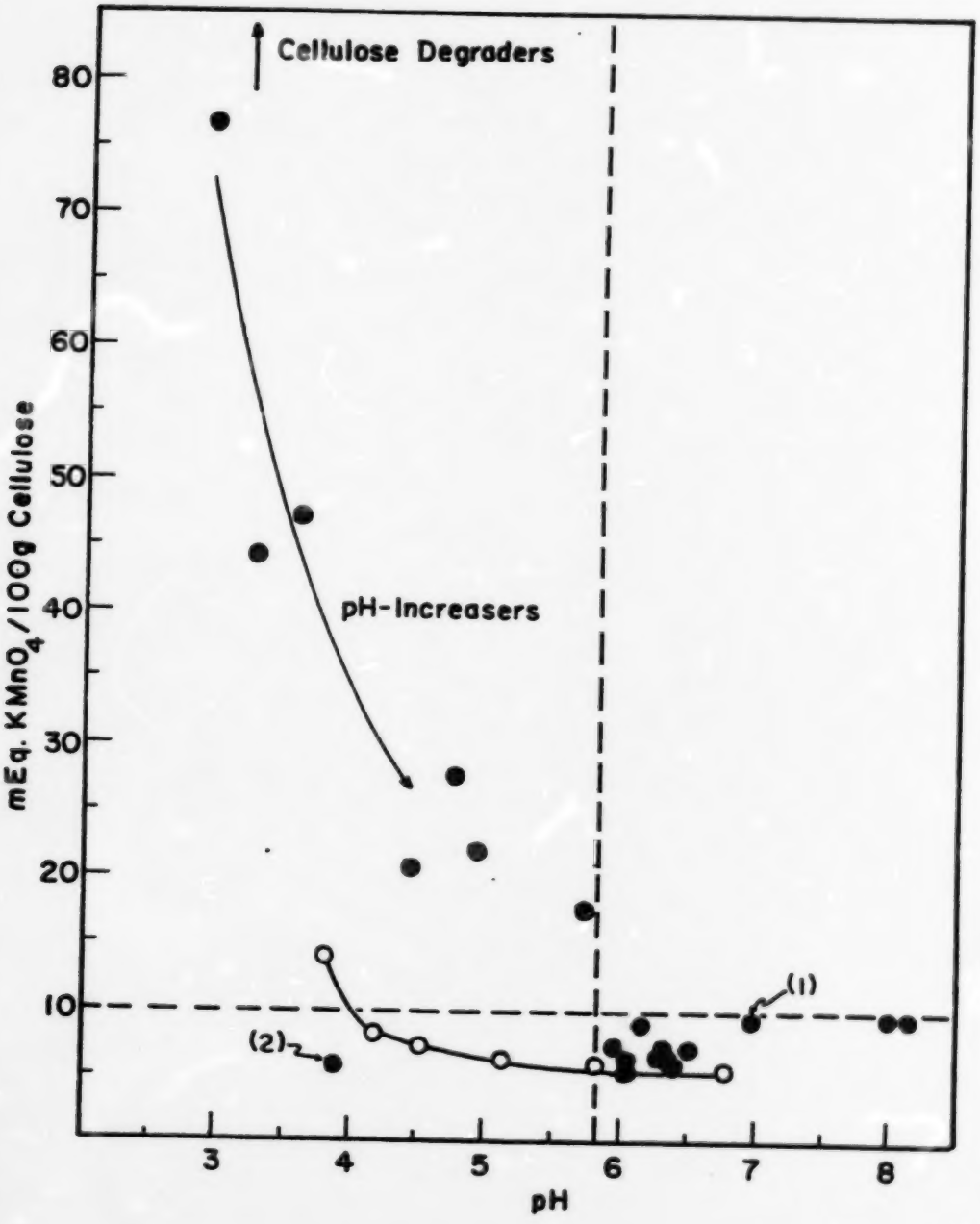

Fig. 7 - Permanganate oxidation of cellulose

• - pH-Increasers
o - Periodate oxidation
(1) - Fresh cellulose
(2) - Cellulose control

Dashed line at pH 5.85 separates significant permanganate-oxidation values from the control range indicated by horizontal dashed lines.

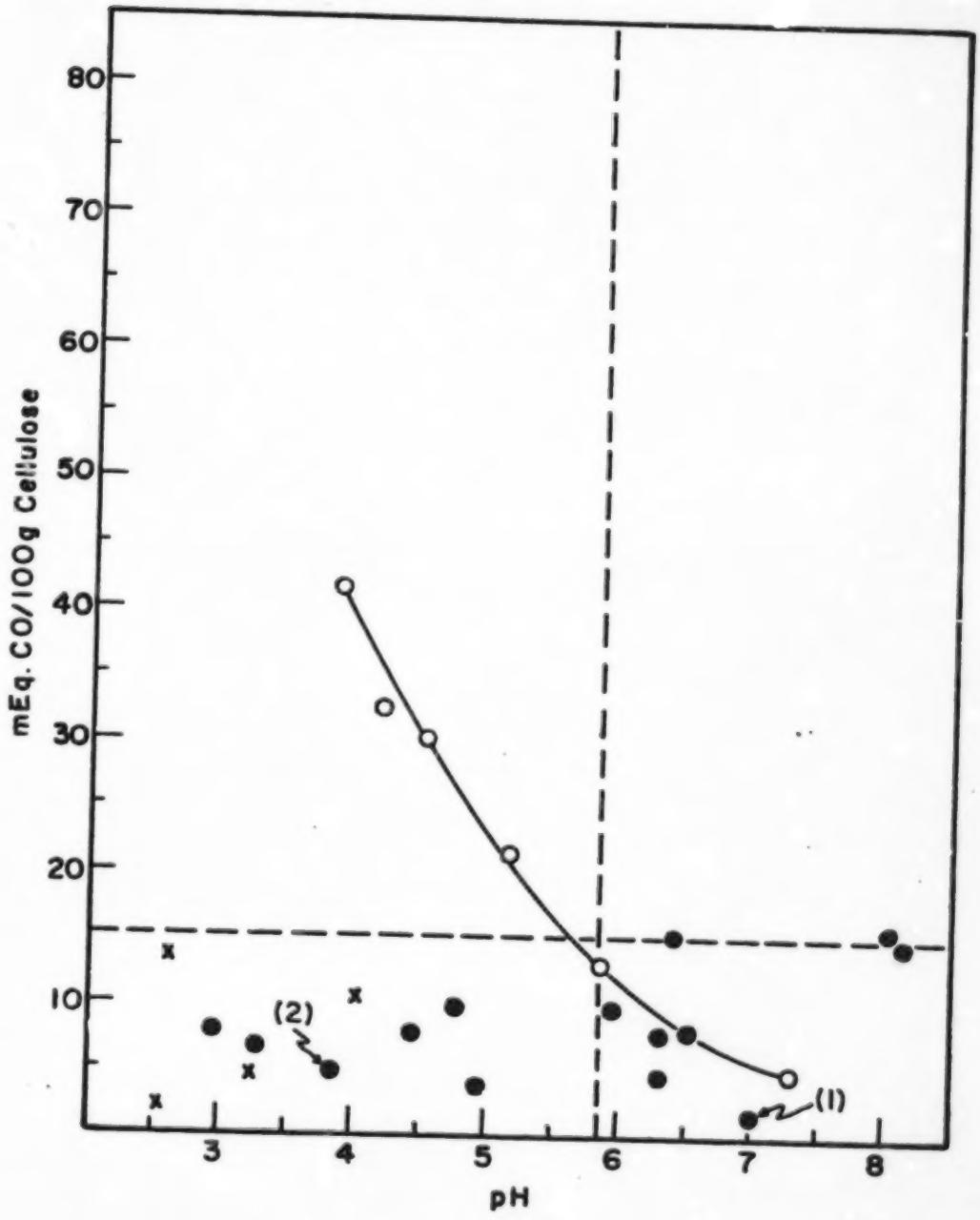

Fig. 8 - Carbonyl contents of cellulose

- ● - pH-Increasers
- x - Cellulose -ydrolyzers
- o - Periodate oxidation

(1) - Fresh cellulose
(2) - Cellulose control

Dashed line at pH 5.85 does not separate carbonyl contents indicated by horizontal dashed line.

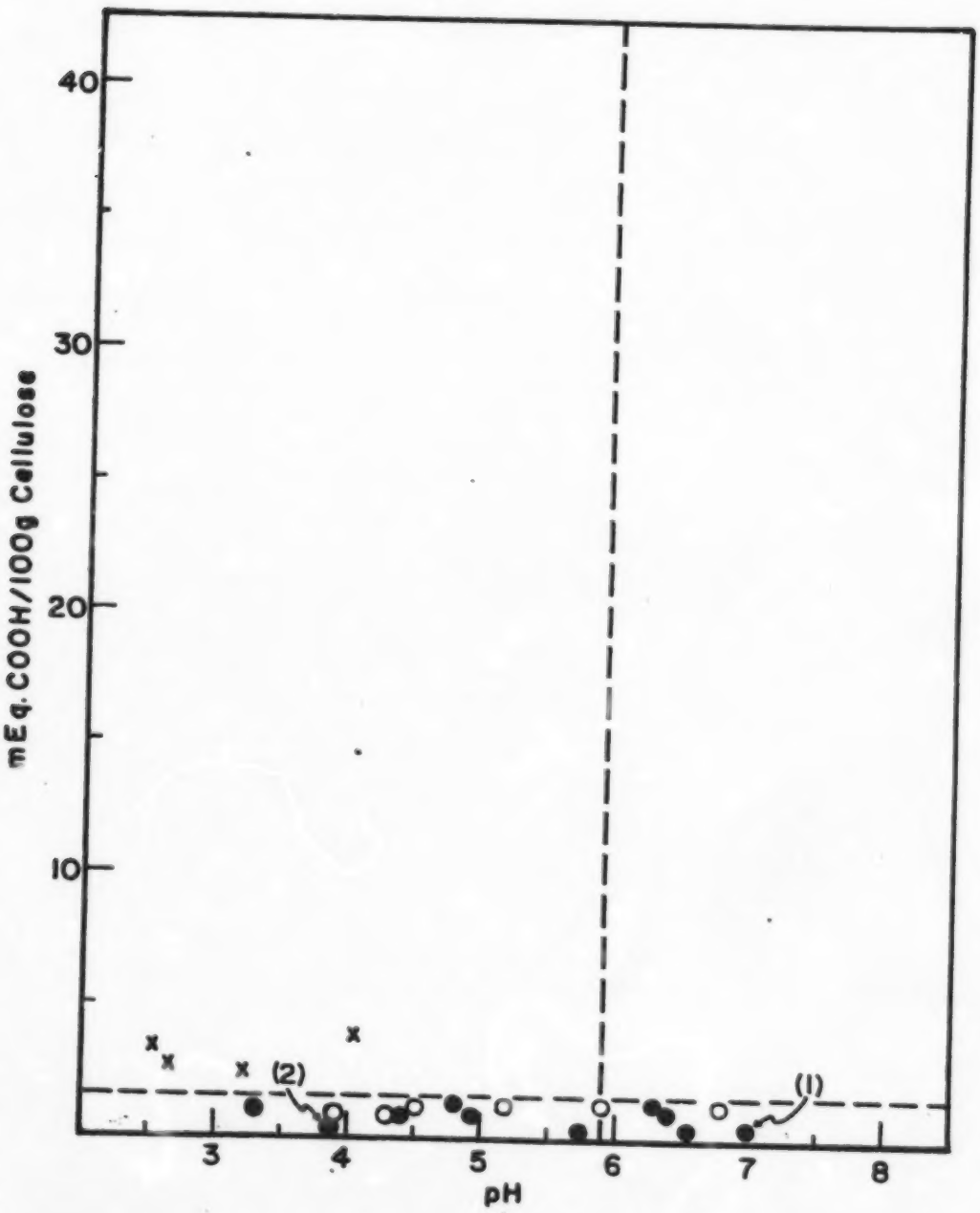

Fig. 9 - Carboxyl contents of cellulose

• - pH-Increasers
x - Cellulose hydrolyzers
o - Periodate oxidation

(1) - Fresh cellulose
(2) - Cellulose control

Dashed line at pH 5.85 does not separate the carboxyl contents indicated by horizontal dashed line.

The compositions of cellulose used by cellulose hydrolyzers are listed in Table 3 showing that the pH had decreased from its initial value. Furthermore, the colour of the cellulose had turned light-brown as compared with cellulose-white unaffected by pH-increasing action. Reduced in volume, the cellulose had become gummy and acquired a resin-type smell.

Table 3 - Analyses of cellulose used by cellulose-hydrolyzing species

Culture	pH		'NaHSO$_3$'	'KMnO$_4$'	Carbonyl	Carboxyl
No.	Initial	Final	mEq/100 g Cellulose			
23-6(8)	4.50	2.53	0.6	126.2	2.5	2.4
23-6(10)	3.85	2.66	0.6	101.2	13.8	2.8
23-6(11)	4.50	4.03	2.1	490	10.5	4.0
23-6(12)	3.85	3.21	0.6	153.9	3.4	2.5

The results of the qualitative chromatographic analyses of solutions from spent-cellulose suspensions are listed in Table 4. Only the solutions from cellulolytic species contained significant soluble metabolites (non-ionic substances such as sugars).

DIFFERENTIATION OF MICROBIAL SPECIES

The results of the selection and differentiation of the pH-increasing and cellulose-hydrolyzing species are listed in Table 5.

Carbohydrate reactions producing acid conditions have been indicated with "+", alkaline conditions with "-alk" and those remaining neutral with "-".

In other tests "+" means "positive" and "-" "negative". Weak or slow reactions have been indicated with (). Microscopically insignificant motility in the "hanging drop", yet producing a marked stab-widening in the test medium has also been indicated with (). Results depending on the medium composition have been indicated with "v".

The growth of all species was generally more abundant on trypticase-soy - than on nutrient agar. Although no experiments were done to provide specific information, the general observation was made that the extent of pH-increasing or cellulolytic properties of the bacteria in the cellulose media

Table 4 - Chromatographic indication of soluble metabolites

Species/ Culture No.	pH		Ion-exchange resin fractions			
	Initial	Final	"Amino-acids"	Non-ionic substances	Organic acids	
					2.5N formic acid	6N formic acid
Aeromonas	3.85	6.0	-	-	-	-
Pseudomonas-I	3.85	6.2	-	-	-	-
Pseudomonas-II	3.85	6.2	-	-	-	-
Achromobacter	3.85	6.1	-	tr	-	-
Flavobacter	3.85	6.0	-	-	-	-
Bacillus	3.85	6.2	-	tr	-	-
23-6(8), cellulolytic	4.50	2.53	-	+	-	-
23-6(10), cellulolytic	3.85	2.66	-	+	-	-
23-6(11), cellulolytic	4.50	4.03	+	+	-	-
23-6(12), cellulolytic	3.85	3.21	-	+	-	-

tr - trace

Table 5 - Properties of selected microbial species

Species	Aeromonas	Pseudo-monas-I	Pseudo-monas-II	Achromo-bacter	Flavo-bacter	Bacillus	"Cellulo-monas-I"	"Cellulo-monas-II"	Micro-cyclus
Culture No.	29-7(1)	29-7(2)	29-7(3)	29-7(4)	29-7(6)	29-7(5)	22-9(1)	22-9(3)	22-9(4)
Morphology	Coccoid Rods 0.9x0.6	Rods 1.5x0.55	Rods 1.45x0.5	Coccoid Rods 1.35x0.7	Coccoid Rods 0.95x0.5	Rods 1.65x0.55 Endospores	Rods 3.1x0.9	Rods 2.85x1.1	Rods, cirles, helical forms
Motilility	+	+	+	-	-	+	v	-	v
Flagella	polar	polar	polar	-	-	lateral	polar	-	polar
Gram	-	-	-	-	-	+	-	-	-
Dextrose oxidation	+	+	- alk	(+)	(+)	(+)	+	+	+
Dextrose fermentation	+	-	-	-	(+)	(+)	+	+	+
Oxidase	v	+	+	+	(v)	-	v	v	v
Catalase	+	+	+	+	+	+	+	+	+
O.N.P.G.	+	-	-	+	+	-	+	-	-
Arginine dihydrolase	-	+	-	-	-	-	-	-	-
Lysine decarboxylase	+	-	-	-	-	-	-	-	-
Ornithine decarboxylase	+	-	-	-	-	-	-	-	-

Table 5 (Cont'd)

Species Culture No.	Aeromonas 29-7(1)	Pseudo-monas-I 29-7(2)	Pseudo-monas-II 29-7(3)	Achromo-bacter 29-7(4)	Flavo-bacter 29-7(6)	Bacillus 29-7(5)	"Cellulo-monas-I" 22-9(1)	"Cellulo-monas-II" 22-9(3)	Micro-cyclus 22-9(4)
Tryptophane deami-nase	-	-	-	-	-	-	-	-	-
Gas from dextrose	+	-	(+)	-	-	-	-	-	-
H_2S Production	-	(+)	-	-	-	-	-	-	-
Gelatin liquefaction	+	+	-	-	(+)	+	-	+	-
Urea	-	-	-	-	-	-	-	-	-
Indole	-	-	-	-	-	-	-	-	-
V.P.	+	-	-	+	-	+	-	-	-
Nitrate reduction	+	-	+	-	-	+	-	-	-
Arabinose	+	+	- alk	-	-	-	-	-	-
Galactose	+	+	-	(+)	-	(+)	-	+	+
Rhamnose	-	- alk	- alk	-	- alk	- alk	- (alk)	- alk	-
Xylose	F +; O alk	+	F +; O alk	(+)	-	-	-	+	-
Mannose	F +; O alk	- alk	F +; O alk	(+)	-	(+)	-	+	-
Lactose	F +; O alk	- alk	- alk	(+)	F alk; O+	(+)	-	+	+
Maltose	+	- alk	- alk	(+)	-	-	-	+	-

Table 5 (Cont'd)

Species Culture No.	Aeromonas 29-7(1)	Pseudo-monas-I 29-7(2)	Pseudo-monas-II 29-7(3)	Achromo-bacter 29-7(4)	Flavo-bacter 29-7(6)	Bacillus 29-7(5)	"Cellulo-monas-I" 22-9(1)	"Cellulo-monas-II" 22-9(3)	Micro-cyclus 22-9(4)
Melibiose	+	-	- alk	-	- alk	- alk	- alk	- alk	-
Sucrose	F +; O -	- alk	- alk	-	-	- alk	- (alk)	- alk	-
Raffinose	F +; O alk	- alk	- alk	(+)	+	-	-	+	+
Trehalose	+	- alk	- alk	(+)	-	-	+,	+	+
Inulin	F +; O alk	- alk	- alk	-	-	-	+	+	+
Adonitol	+	- alk	- alk	(+)	-	-	-	-	-
Dulcitol	F +; O alk	- alk	- alk	-	-	-	-	-	-
Mannitol	F +; O alk	- alk	- alk	-	-	-	- (alk)	- alk	-
Sorbitol	+	- alk	- alk	- alk	- alk	- alk	- (alk)	- alk	-
Glycerol	+	- alk	- alk	-	-	+	+	+	-
Inositol	+	- alk	- alk	- alk	- alk	- alk	- alk	- alk	- alk
Amygdalin	-	- alk	- alk	-	-	- alk	- alk	- alk	-
Salicin	+	- alk	- alk	(+)	(+)	-	+	+	-
Citrate	+	+	+	-	-	+	-	-	-
Acetate	F -; O -alk	- alk	-	- alk	- alk	- alk	- (alk)	- alk	-
Malonate	F -; O -alk	- alk	- alk	- (alk)	-	(alk)	-	-	-

Table 5 (Cont'd)

Species	Aeromonas	Pseudo-monas-I	Pseudo-monas-II	Achromo-bacter	Flavo-bacter	Bacillus	"Cellulo-monas-I"	"Cellulo-monas-II"	Micro-cyclus
Culture No.	29-7(1)	29-7(2)	29-7(3)	29-7(4)	29-7(6)	29-7(5)	22-9(1)	22-9(3)	22-9(4)
ß-Hydroxy-butyric acid	+	+	+	-	-	-			
Casein hydrolysis	+	+	-	-	-	+			
Starch hydrolysis	-	-	-	-	-	+	+	+	+
Cellulose hydrolysis	-	-	-	-	-	-	+	+	+
Butane-diol	+	-	-	-	-	+			
Ps. isolation agar	+	+	+	-	-	-			
Tetrazolium in starch	(+)	-	-	+	+	+	+	+	+
Tetrazolium in cellulose	-	-	-	(+)	-	(+)	+	+	+
pH-increase	+	+	+	(+)	(+)	(+)	-	-	-

F = Fermentative
O = Oxidative
alk = alkaline
() = slow or weak
v = variable

depends very much on synergistic relationships between the various species. Uniformity of agar-colony morphology appeared not necessarily indicative of culture purity. Several species in these relationships may even lack pH-increasing or cellulolytic properties, yet play a role in the ecology (40). The selected species do, therefore, not represent the complete composition of the actual microbial population.

DISCUSSION AND CONCLUSIONS

ORIGIN OF THE pH-INCREASE

The pH-increase in nutrient cellulose-suspensions is due to microbial action as the pH of a control did not change. The lower limit of the microbial action is approximately pH 3.5 (Fig. 1).

Wood-degrading fungi can contribute to the pH-increase, preferring wood over cellulose (Fig. 2). A fungus and a bacterial isolate, selected for their fast pH-increases, used cellulose instead of its degradation product, glucose, for increasing the pH (Fig. 3). Because this and other bacterial isolates did not disintegrate filter paper or cleared cellulose agar, the origin of the pH-increasing action appears to be connected with cellulose. Cellulolytic isolates, however, decreased the pH (Table 3). The primary action of the pH-increasers is possibly similar to that of Cellvibrio or Cellfalcicula attributed to "species incertae sedis" (41). These species did not disintegrate cellulose either, but produced oxy-cellulose (42). The primary pH-increase may originate, therefore, in a similar bacterial action so that analyses of spent cellulose would contribute information about this process.

A secondary pH-increase may be produced by species that develop alkaline conditions in carbohydrate tests (Table 5). To what extent this takes place is not known because acid production from carbohydrates is also present.

INFORMATION USEFUL FOR A PRACTICAL PROCESS DESIGN

1. The cellulose-degradation products and pH-increase required for activating sulphate-reducers in acid mine water originate in the action of, at least, two different groups of microbial species, cellulose-hydrolyzers and pH-increasers.

(a). The action of cellulose hydrolyzers is typified by a reduction (see tetrazolium, Table 5) producing often a pH-decrease and a transformation of cellulose into a gummy substance having a resin-type smell. The reduction results in a permanganate oxidation much higher than that of cellulose used by pH-increasers (Table 3, Fig. 7). These cellulolytic species produced also more soluble metabolites (Table 4).

The action of pH-increasers at low pH may be similar because a pH-decrease (Fig. 1) took place before the sustained increase. This was also suggested by high permanganate-oxidation values, e.g., 76.6 mEq $KMnO_4$/100 g cellulose obtained from a pH-increaser (Pseudomonas-I) at decreased pH (2.95). The tetrazolium reduction was nil or significantly less than that of the cellulose hydrolyzers. Weak reactions from pH-increasers may have been the result of an action during a temporary pH-decrease (Fig. 1) similar to that of the latter. It is possible that the pH-increasing or decreasing action is connected with metabolic properties sensitive to environmental conditions (see results of oxidase reaction or motility under "Microbial Differentiation"). However, the problem if and to what extent one species can act either way depending on environmental (nutritional) conditions was not investigated because the study was aimed at practical applications of the process and the species were differentiated near the end.

(b). The action of pH-increasers on cellulose results in, at least, two processes as indicated by the relationships between the pH and the cellulose-bisulphite addition (Fig. 6), permanganate-oxidation (Fig. 7) and chloride-content (Fig. 5). In the low pH-range, between approximately 3.5 and 6, permanganate oxidation of spent cellulose decreases while the Cl-uptake increases with the pH. Bisulphite-addition does not take place here, but in the pH-range above 6 where Cl-uptake has decreased and permanganate oxidation has become negligible. The oxidation, indicated by the bisulphite-addition in this range as compared with the low pH-range, is specific and limited to the formation of double-bonded aldehyde-, aldol- or ketone-type oxygen (43,13). The carbonyl determinations did not indicate a significant relationship with the pH, and the carboxyl contents were negligible. Chemical oxidation by periodate, decreasing the pH, increasing the bisulphite-addition slightly (Fig. 6) but the carbonyl contents significantly (Fig. 8), was different from the microbial action.

The extrapolation of the cellulose-chloride uptake (Fig. 5) from pH 3.7 to pH 7 would indicate a cellulose-chloride content to be expected by a theoretical exchange between Cl^- ions in the solutions and OH^- ions of the cellulose (see Appendix 1). Thus, it is tempting to attribute the pH-increase in the 3 to 6-pH range to such a mechanism. However, in view of the complexities in cellulose chemistry and microbiology, conclusions in connection with relevant reaction mechanisms require further experimentation.

2. In the practical process application, the different properties of the cellulose hydrolyzers and pH-increasers require a separation of environmental conditions in the woodwaste mass. A limited oxidation, possibly in the form of aeration, should be applied not disturbing the woodwaste structure, and alternating with locations favourable for a reduction process. Therefore, acid mine water or effluent from an abandonned tailings area, having its flow-rate controlled by a dam (weir), could be treated with woodwaste in a channel emptying into a series of settling lagoons. Here, sulphate-reducing organisms would precipitate heavy metals as sulphides. The channel could contain a series of baffles consisting of two plates leaving a narrow space devoid of woodwaste for aeration. One plate would be equipped with an opening at the bottom, and the other would produce an overflow. This would create conditions favourable for microbial action at low pH (reduction) at the bottom of the baffles and at pH-values above 6 (oxidation) in the overflow. Overflow and dredging could be used in the lagoons for water purification.

THE BACTERIAL SPECIES

The microbial population of the wood decaying in the acid tailings included a great number of species that did not increase the pH nor degrade cellulose. A few species were selected, however, to indicate the variety directly connected with the cellulose process. The differentiation was limited to attributing the species tentatively to genera, thus typifying the species. It did not intend to be a taxonomic study.

The attribution of the species in culture No. 29-7(1) to the genus Aeromonas was based on the polar-flagellated rods having Gram-negative, dextrose oxidative and fermentative properties. Furthermore, the species produced gas (CO_2) and had a positive butane-diol test. However, instead of arginine dihydrolase, it has lysine- and ornithine-decarboxylase actions. The motility in a trypticase-soy medium was significantly greater than that in a starch medium. The oxidase test was also dependent on the nutrient composition.

Although the polar flagellates in culture No. 29-7(2) were also Gram-negative and oxidative in dextrose, they were not fermentative. The species was oxidase positive and arginine-dihydrolase positive. It used β-hydroxy-butyric acid and was, therefore, attributed to the genus Pseudomonas-I. Although typified as a pH-increaser, the opposite action was also observed.

The rods in culture No. 29-7(3) were also tentatively attributed to the same genus, Pseudomonas-II. In addition to lacking fermentative action on dextrose, it was oxidase positive and used β-hydroxy-butyric acid. It has, however, alkaline action in dextrose and a positive nitrate-reduction test. Both species may be related to P. alcaligenes because of their alkaline action on several carbohydrates.

The non-motile rods in culture No. 29-7(4) were attributed to the genus Achromobacter (44). It was Gram-negative, oxidative in dextrose and oxidase positive. Because of its non-motility it could not be attributed to the genus Alcaligenes (41). Furthermore, it was slightly fermentative after two weeks in glucose and lactose, affected litmus-milk only after four weeks, and produced slowly acid in several carbohydrates. Its methyl-red test was negative. The tentative typification as an achromobacter was preferred over that of a pseudomonad (41).

The species in culture No. 29-7(5) was attributed to the genus Bacillus because it was Gram-positive and produced endospores. Long flexible rods could be obtained when grown in nutrient broth. Staining showed, however, that the rods contained several short cells. Its colonies were slimy on most agars. Lateral flagella developed after one or more polar shown by young cells. The spores did not distend the sporangium distinctly and had a central to terminal position.

The species in culture No. 29-7(6) produced yellow coloured colonies. Its fermentative action in dextrose was negative after 72 h, but turned slowly positive after another few days. The oxidase test was slowly positive in trypticase-soy, but negative on starch agar. Being non-motile and Gram-negative it was attributed to the genus Flavobacter.

All the foregoing species increased the pH. None of them cleared cellulose agar, disintegrated filter paper or reduced tetrazolium significantly. The properties of the following species are the opposite in these respects. They are cellulolytic and reduce tetrazolium, rather increasing than decreasing the water acidity. Tetrazolium did not inhibit microbial growth.

The rods in culture No. 22-9(1) and 22-9(3) are typified by the name "Cellulomonas" because of their cellulolytic, dextrose-oxidative and fermentative actions. The Gram-negative rods, however, lacked the coryne-form-type of morphology. Strains of culture No. 22-9(1) differed in cell morphology. However, they produced the same test results as listed in Table 5. Motility in a starch medium was not as great as that in nutrient broth. The oxidase test was negative in a starch-, but positive in a trypticase-soy medium. The species is listed as Gram-positive in Buchanan and Gibbons' "Manual" (41), although the difficulty to obtain this reaction is acknowledged in the description. Because of its negative Gram-reaction and cell morphology the species is probably related to the following "Microcyclus". These species suggest the presence of a group of Gram-negative, dextrose oxidative and fermentative polar-flagellates reducing or oxidizing ("Aeromonas") cellulose.

The species in culture No. 22-9(4) resembles species of the genus Microcyclus in morphology. Its rods produce circles, horshoe-, helical- and other convoluted forms. The genus Myconostoc described originally with polar flagellated swarm-cells (44) has been included in that of Microcyclus (41) because of curved and spiral chains. Its taxonomic position is, therefore, based to a great extent on its morphology. However, the development of ring and helical forms of the "22-9(4)" species depends on the type of nutrient medium, being more pronounced in starch- than in nutrient-, dextrose or trypticase-soy agar. The curvature of the cells may, therefore, originate in a lack of essential nutrient required to sustain cell growth along the rod axis. Although the ring and helical-shaped cells were non-motile, the straight, curved and even part of the horshoe and irregularly twisted rods were motile with polar flagellation. The cellulolytic action, however, was slow. These properties and the dextrose-fermentative action deviate from those of the genus Microcyclus (45). The oxidase test results were similar to those of cellulomonads. Curved, Gram-negative, polar flagellated rods acting on cellulose have been described as Cellvibrio and Cellfalcicula (44), but were attributed as "species incertae sedis" to the genus Pseudomonas (41). Because of its fermentative action the "22-9(4)" species could be typified as a "microcyclic vibrio".

The cellulolytic species were difficult to preserve particularly in liquid media. Cellulose and starch-agar slants were used for this purpose, but were still not satisfactory. Synergistic relationships may fulfill unknown metabolic requirements.

A species that was not included in the experiments because of its slow growth, was attributed to the genus Micromonospora. Its folded orange colonies on starch agar appeared to consist of a mycelium producing single spores. It had slow cellulolytic action. To what extent this species increases the pH is not known.

ACKNOWLEDGEMENTS

Thanks are due to D.W. Stranks and J.F. Shields of Forintek Canada Corporation, formerly Eastern Forest Products Laboratory, Environment Canada, for supplying several species of wood-degrading fungi from their collection; to J.D. Cooney and G. Colin of the Textile Chemistry Section, National Research Council, for their comments and suggestions on the manuscript.

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APPENDIX 1

CELLULOSE-CHLORIDE CONTENT PRODUCED BY THEORETICAL ANION-EXCHANGE AT pH 7

A process involving concentrations of both cellulose and nutrient chloride-ions may be indicated generally by the reaction:

$$[H^+] + [\text{Cellulose-OH}] + [Cl^-] \rightarrow [\text{Cellulose-Cl}] + [H_2O]$$

Hydroxyl- and chloride ion concentrations are constant in solutions of identical composition and pH-value. Increases in cellulose concentration do not affect the principle of the reaction because most of the 'Cellulose-OH' is not used in the exchange with chloride ions and the cellulose-chloride concentration appeared independent of the cellulose concentration, at least, in the range of 0.2-0.4% cellulose (p 16). A surplus of chloride ions need not be considered because of the low concentrations compared with those of cellulose.

Should the process involve an ion-exchange mechanism, the interaction between pH and cellulose-chloride concentrations can be explained as follows. A change from pH 3.5 to 7.0 would involve $[10^{3.5} - 10^{7.0}]$ grammoles OH^--ions exchanging with the same amount of Cl^--ions per litre, or $[10^{0.5} \times (10^{-4} - 10^{-7.5})] \times 35.46$ g Cl/L = 11.21 mg Cl/L. The experiment (Fig. 5) used 20 g cellulose/L; thus a cellulose-chloride concentration of $\frac{11.21}{20}$ mg Cl/g cellulose = 561 µg Cl/g cellulose would be expected at pH 7. According to the relationship in Fig. 5 the chloride concentration is approximately 23 µg Cl/g cellulose. This, however, is caused by a cellulose-chloride decreass from a maximum reached at pH 5.5 or higher (up to pH 8.5).

An increase in the concentrations of cellulose-chloride can be expected from initial pH-values less than 5.5, if the pH-increase is an ion-exchange process. On extrapolation (Fig. 10) the cellulose-chloride concentration at pH 7.0 is: 875 (i.e., $10^{2.92}$) µg Cl/g cellulose. The theoretical value of 561 (i.e., $10^{2.75}$) µg Cl/g cellulose suggests that an ion-exchange process (or esterification) might be a result of the bacterial action increasing the nutrient-pH from 3.5 to at least 5.5.

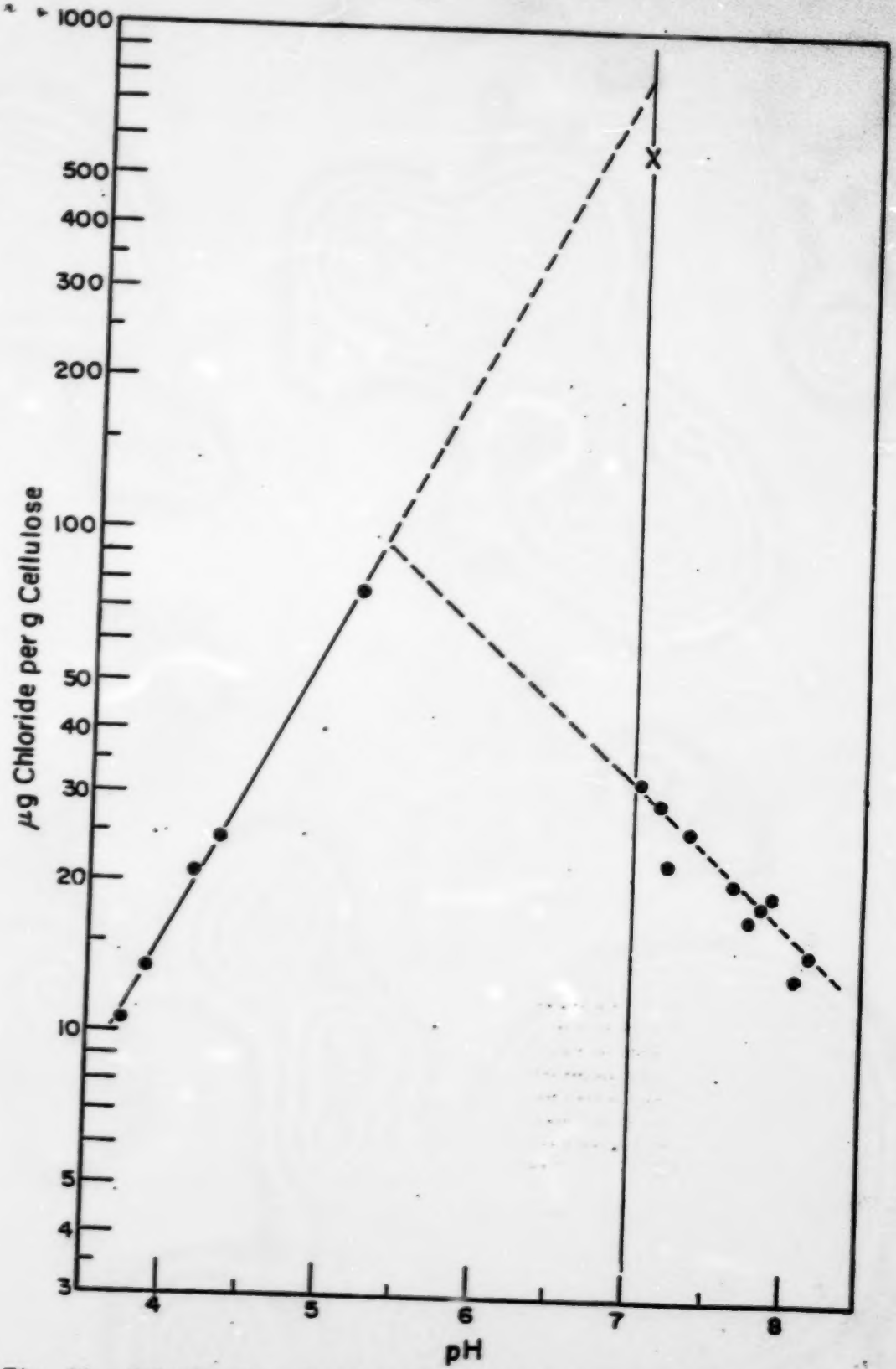

Fig. 10 - Relationships between pH-values and the cellulose-chloride concentrations

x - Cellulose-chloride content, 561 µg Cl/g cellulose produced by theoretical anion-exchange

● - Empirical relationships from Fig. 5